Effect of Plant Sterols and Tannins on *Phytophthora ramorum* Growth and Sporulation

Rachel A. Stong · Eli Kolodny · Rick G. Kelsey ·
M. P. González-Hernández · Jorge M. Vivanco ·
Daniel K. Manter

Abstract Elicitin-mediated acquisition of plant sterols is required for growth and sporulation of *Phytophthora* spp. This study examined the interactions between elicitins, sterols, and tannins. Ground leaf tissue, sterols, and tannin-enriched extracts were obtained from three different plant species (California bay laurel, California black oak, and Oregon white oak) in order to evaluate the effect of differing sterol/tannin contents on *Phytophthora ramorum* growth. For all three species, high levels of foliage inhibited *P. ramorum* growth and sporulation, with a steeper concentration dependence for the two oak samples. *Phytophthora ramorum* growth and sporulation were inhibited by either phytosterols or tannin-enriched extracts. High levels of sterols diminished elicitin gene expression in *P. ramorum*; whereas the tannin-enriched extract decreased the amount of 'functional' or ELISA-detectable elicitin, but not gene expression. Across all treatment combinations, *P. ramorum* growth and sporulation correlated strongly with the amount of ELISA-detectable elicitin (R^2=0.791 and 0.961, respectively).

R. A. Stong · E. Kolodny · D. K. Manter (✉)
USDA-ARS, Soil-Plant-Nutrient Research, Fort Collins, CO, USA
e-mail: daniel.manter@ars.usda.gov

R. G. Kelsey
USDA Forest Service, PNW Research Station, Corvallis,
OR, USA

M. P. González-Hernández
Department Crop Production, Santiago de Compostela University,
Lugo, Spain

J. M. Vivanco
Center for Rhizosphere Biology, Department of Horticulture
and Landscape Architecture, Colorado State University,
Fort Collins, CO, USA

Introduction

Elicitins, a unique class of elicitors, make up a family of proteins secreted by members of the phytopathogenic Chromista, *Phytophthora* spp. and *Pythium* spp. (Kamoun et al. 1993; Panabieres et al. 1995; Vauthrin et al. 1999). Elicitins play an important role in the success of *Phytophthora* spp. infection and growth. In non-host species, elicitins can trigger a successful hypersensitive response (Pernollet et al. 1993; Vleeshouwers et al. 2000). In host species, the potential role of elicitins in disease development is incompletely understood. However, *Phytophthora ramorum* elicitins are linked to increased pathogen virulence (Manter et al. 2010), photosynthetic declines in tanoak, rhododendron, and bay laurel (Manter et al. 2007b), but not beech (Fleischmann et al. 2005), and ultra-structural changes in oak (Brummer et al. 2002) and pepper (Ivanova and Singh 2003).

One of the main biological functions of elicitins for *Phytophthora* spp. appears to be sterol binding and transfer through phospholipid membranes (Mikes et al. 1998; Vauthrin et al. 1999). *Phytophthora* spp. lack the ability to synthesize sterols, but sterols are required for sexual and asexual reproduction (Elliott et al. 1966; Haskins et al. 1964; Hendrix 1970; Leal et al. 1964; Nes and Stafford 1983). As a result, *Phytophthora* spp. take up and metabolize a variety of sterols from host plants (Gonzalez and Parks 1981; Grant et al. 1988; Nes and Stafford 1983, 1984). Sporulation rates differ depending upon the sterol acquired (Nes et al. 1980; Nes and Stafford 1983, 1984).

Due to the discrimination of *Phytophthora* spp. against various sterols and the associated changes in growth and spore formation, one might expect plants with quantitative and/or qualitative differences in plant sterol content to exhibit variable field resistance and/or to influence sporulation rates of *Phytophthora* spp. In a study with various potato cultivars, Hazel et al. (1988) examined *P. infestans* sporulation on both detached potato leaves and artificial

media amended with a mixture of sterols designed to mimic the potato leaf sterol profiles. Although sterol treatment did increase sporangia production compared to non-sterol treatments, and sporangia production differed significantly between the potato cultivars, the *in planta* and *in vitro* treatments were not well correlated, suggesting that additional plant compound(s) influenced sterol acquisition.

Phytophthora ramorum is a highly virulent pathogen that infects diverse hosts including deciduous, evergreen, woody, and herbaceous plants (Goheen et al. 2006; Grunwald et al. 2008). The result of infection varies depending on the plant host species, tissue type, and isolate of *P. ramorum* (Goheen et al. 2006; Grunwald et al. 2008; Manter et al. 2010; Rizzo et al. 2005). Factors influencing host resistance are largely unknown, although various phytochemicals have antimicrobial activity against *P. ramorum*. For example, three terpenes from conifer heartwood had strong antimicrobial activity against *P. ramorum* (Manter et al. 2007a). A study of phloem tissue in coast live oaks found significant differences between infected and non-infected tissue for several phenolic compounds, including gallic acid, catechin, and ellagic acid (Nagle et al. 2011; Ockels et al. 2007).

One class of compounds that may influence sterol acquisition via elicitins is the group of polyphenolic compounds known as tannins. Tannins play a role in plant resistance by exhibiting direct toxicity against a wide variety of microbes (Latte and Kolodziej 2000; Nelson et al. 1997; Sivakumaran et al. 2004) although the mechanistic basis for this activity is not well described. One plausible mechanism, may be associated with the ability of tannins to bind and precipitate proteins (Hagerman and Butler 1981; Shahidi and Naczk 1995) that are necessary for pathogen growth and survival, such as the *Phytophthora* elicitin protein.

The goal of this *in vitro* study was to explore the potential effect sterols and/or tannin-amended artificial growth media may have on *P. ramorum* growth, sporulation, and elicitin production. The leaf tissue, sterol, and tannin extracts were obtained from three different plant species, California bay laurel, California black oak, and Oregon white oak (*Umbellularia californica*, *Quercus kelloggii*, and *Q. garryana*) in an effort to obtain extracts with differing quantities and/or compositions of sterols and tannins.

Methods and Materials

Pathogen Strain and Plant Material A single North American isolate (PR-07-031, original name 15-WA-M isolated from soil in Washington 2006) of *P. ramorum* was grown as a starter culture for sporulation experiments on corn meal agar (Becton Dickinson, Rutherford, NJ, USA) without light at room temperature. A single 0.7 cm agar plug

was taken from the outermost margins of 2–3 week-old starter cultures and used to inoculate agar (15 g l^{-1}, Bacto agar, Becton Dickinson) plates containing the *Phytophthora* synthetic medium (PSM) formulated by Hoitink and Schmitthenner (1974) and amended with various amounts of ground leaf tissue, sterols, and/or tannin-enriched extracts in lieu of the recommended cholesterol (10 mg l^{-1}). All treatments were replicated three times in each of three independent trials.

Leaf Tissue Preparations Foliage for sterol extraction was collected from trees in Oregon between 29 September and 4 October, 2007. Leaves of Oregon white oak were gathered from randomly sampled branches of several native trees in Philomath, Benton County (44° 32′ 21.42″N, 123° 20′ 20.30″W, elevation 108 m). California black oak foliage was sampled from several trees growing near Jacksonville, Jackson County (42° 15′ 48″N, 122° 59′15″, elevation 824 m), and leaves of California bay laurel were gathered from a single ornamental tree in Albany, Linn County (44° 37′ 22.25″N, 123° 5′ 47.58″W, elevation 70 m). The leaves were removed from the stems, air dried in the laboratory, and stored at room temperature until needed.

Foliage used to amend culture media, or for tannin extraction, was gathered on 9 September, 2008 from the same Oregon white oak and bay laurel trees sampled in 2007. However, a new black oak tree was sampled near Eugene (Lane County, 44° 0′ 40.59″N, 123° 4′ 59.08″, elevation 144 m). After collection, foliage was allowed to air-dry at room temperature for 7 d before grinding with a Wiley-mill, sieving with a 5 mm mesh screen, and storing at 4 °C until needed. Ground leaf tissue was added directly to PSM prior to autoclaving at a final concentration of 0.1, 0.5, 1.0, or 5.0 mg ml^{-1}.

Commercially Available Sterols Several commercially available sterols—β-sitosterol, cholesterol, ergosterol, stigmasterol, and stigmastanol (Sigma-Aldrich, St. Louis, MO)—were selected for their variable properties. β-Sitosterol is the most common plant sterol, and is structurally similar to cholesterol except for the ethyl substitution at position 24. Although the quantity of cholesterol in plants typically is low in terms of total lipid content, it is a frequent component of plant membranes, and it may be the major sterol on leaf surfaces (Behrman and Gopalan 2005). Stigmasterol is the second most common plant sterol and is nearly identical to β-sitosterol except for the absence of a double bond. Ergosterol is similar to cholesterol but is almost exclusively found in fungi. All sterols were dissolved in ethanol and added directly to PSM prior to autoclaving at a final concentration of 0.1, 1, 10, 25, or 50 μM.

Plant Sterol Extracts Leaf sterols were extracted according to the methods of Jeong and Lachance (2001). Air-dried, ground leaf tissue (10 g) was placed in a screw-cap 250 ml

glass bottle before adding 25 ml of 50 % KOH and 100 ml 95 % ethanol and heating in an 80 °C water bath for 1 hr. The solution was transferred to a separatory funnel with the aid of 30 ml 95 % ethanol, 50 ml warm distilled water, followed by 50 ml cold water. The mixture was rinsed six times with 100 ml petroleum ether. The combined petroleum ether fraction was divided into two aliquots (300 ml) and each was washed 4 times with 100 ml distilled water. The petroleum ether fractions were combined and concentrated to less than 50 ml in a rotary evaporator at 40 °C. Anhydrous sodium sulfate (1.0 g) was added, and the solution was transferred to a weighed 50 ml round bottom flask with the aid of 5 ml methylene chloride, air-dried, and reweighed. Residual water in the air dried tissue was determined by mass using triplicate subsamples of ground tissue (250 mg) dried 16 hr at 102 °C. The sterol extract yield was calculated on a dry mass basis.

The concentrated bay laurel extract retained a strong odor from monoterpenes, and the presence of these contaminants was confirmed by gas chromatography. These volatiles were removed before testing the extract on *P. ramorum*, as they can be inhibitory. The extract was redissolved in methylene chloride, and a portion was transferred to a small round-bottom flask before removing 70 % of the solution on a rotary evaporator at 40 °C. Monoterpenes were removed by adding 20 ml of dH_2O to the flask and evaporating to dryness in a 70 °C water bath. The removal of the monoterpenes was then verified by gas chromatography, as outlined below.

Sterol concentrations in the extracts were quantified on a Hewlett Packard (HP) 5890 Series II gas chromatograph with an Agilent (J&W Scientific, Inc.) DB-5 column (30 m × 0.25 mm, 0.25 µm film thickness) connected to a flame ionization detector. The helium carrier gas flow rate was 1.0 ml min^{-1} at 150 °C and a split of 1:20. The column temperature started at 150 °C and was increased 5 °C min^{-1} to 300 °C where it was held for 20 min. The injector and detector temperatures were 250 °C. Extracts were dissolved in hexanes, or 2:1 hexanes:methylene chloride (3 to 12 mg ml^{-1}) containing isophytol as an internal standard, and 2 µl were injected. Compounds were quantified with three point standard curves using solutions of the commercial samples dissolved in methylene chloride with isophytol added as an internal standard. Compounds in leaf extracts were identified using the same conditions as above but with an Agilent (J&W Scientific, Inc.) DB-5MS column connected to an HP 5970 mass selective detector. The split was set at 1:10 and extract concentrations increased to about 20 mg ml^{-1}.

The dry plant sterol residues were dissolved in ethanol and added directly to PSM media prior to autoclaving at a final concentration of 1, 5, 10, 50, or 100 µg extract ml^{-1}. Due to the presence of non-sterol components in the extract (Table 1), the actual amount of sterols added to the PSM media differed among samples; therefore, all analyses and reported masses were based on the amount of α- and β-sitosterol in the extract (9.51, 5.08, and 6.92 % of the total sterol extract for bay laurel, black oak, and white oak samples, respectively).

Plant Tannin-enriched Extracts Sub-samples (5 g) of the ground bay laurel, black oak, or white oak foliage were extracted × 3 with 100 ml of 70 % acetone for at least 4 hr at room temperature. Tannin fractions were purified after evaporating the extracts under nitrogen and re-dissolving each extract in 50 ml of ethanol. Ethanol samples were applied to 5 ml Sephadex LH-20 columns that were washed with at least 50 ml ethanol or until absorbance at 280 nm in the effluent was no longer detected, then eluted with 50 ml of 70 % acetone (Strumeyer and Malin 1975) and used directly as described below.

Total tannin content of the LH-20 column eluent was determined chemically and gravimetrically. The Folin-Denis assay (Folin and Denis 1912) was used to assess total phenols, using tannic acid (MP Biomedicals, Solon, OH, USA) as a standard. Condensed tannin was measured using the acid butanol assay (Porter et al. 1986) using procyanidin C1 (PHY89537, Cerriliant, Round Rock, TX, USA) as a standard. The concentration of galloyl-containing compounds (e.g., gallotannins, ellagitannins, and other galloyl esters) was measured using the rhodanine assay (Inoue and Hagerman 1988) with gallic acid as a standard. The remaining LH-20 column eluents were evaporated under nitrogen, weighed, and re-dissolved in ethanol at a final concentration of 10 mg extract ml^{-1}. Tannins were added directly to PSM prior to autoclaving at a final concentration of 0.1, 1, 10, 25, and 50 µg dry mass ml^{-1}.

Phytophthora ramorum Growth and Sporulation Inoculated plates were incubated for 11 d at 18 °C in darkness before measuring average colony diameter (two perpendicular measurements per plate). Plates were then flooded with 8 ml of distilled H_2O to induce sporangium formation, and incubated overnight at 18 °C in darkness. After 18 hr, a 200 µl aliquot of distilled H_2O was removed from each plate and stored at 4 °C for elicitin determination using the ELISA assay described below. Plates were incubated for 2–4 hr at 4 °C before warming to room temperature to stimulate zoospore release. Zoospore solution was poured from each plate into a 17 × 100 mm, 14.0 ml culture tube, and vortexed two times for 20 sec to encyst swimming zoospores. The zoospores were quantified using a hemocytometer at 40× magnification.

ELISA Assay Elicitin concentration was determined with a custom, indirect ELISA assay using rabbit anti-elicitin

Table 1 Foliar sterol and tannin concentrations

Extract	Compound/Assay	Units	Bay laurel	Black oak	White oak
			Concentration in dry matter		
Sterol	Phytol	µg g^{-1}	848	849	851
	Heptacosane	µg g^{-1}	251	363	449
	Nonacosane	µg g^{-1}	481	797	62
	Hentricontane	µg g^{-1}	227	10	12
	α-sitosterol	µg g^{-1}	95	20	11
	β-sitosterol	µg g^{-1}	1,650	592	701
Tannin	Gravimetric	µg mg^{-1}	5	42	20
	Folin-Denis	µg TAE mg^{-1}	34	130	88
	Rhodanine	µg GAE mg^{-1}	8	68	19
	Acid butanol	µg C1E mg^{-1}	4	42	14

TAE tannic acid equivalents

GAE gallic acid equivalents

C1E procyanidin C1 (PHY89537, Cerriliant, Round Rock, TX) equivalents

polyclonal antibodies (Covance Research, Denver, PA, USA) as described previously (Manter et al. 2010). Absorbance at 650 nm was recorded every 30 sec for 15 min with shaking using a Biotek ELx808 microplate reader (Winooski, VT, USA). Elicitin concentrations were determined using an external standard curve (100, 50, 25, 12.5, 6.3, 3.1, 1.6, 0.8, 0.4, 0.2 µM) using a purified recombinant ram-α2 protein (Manter et al. 2010).

Elicitin RT-qPCR Liquid PSM medium, amended with either foliar sterols or tannins at the same concentrations used for the bioactivity assays, was inoculated with *P. ramorum* (PR-07-031) and cultured for 2 wk. Total RNA was extracted from the mycelium using the RNeasy Total RNA Extraction kit (Qiagen, Germantown, MD, USA), and cDNA was synthesized using the RETROscript kit (Applied Biosystems/Ambion, Austin, TX, USA) following the manufacturer's recommendations. Elicitin (ram-α2) gene expression was determined using the primers, TaqMan probe, and procedures outlined in Manter et al. (2010). All reported values of ram-α2 gene expression are relativized to β-tubulin using the standard curve method.

Statistical Analysis The effect of the various foliage, sterol, or tannin amendments on vegetative growth (i.e., colony diameter), zoospore production, ELISA-detected elicitin secretion, and elicitin gene expression were analyzed by nonlinear regression PROC NLIN (SAS, Vers 9.3). For each trial/treatment combination, response curves were fit in order to calculate 3 different parameters ($X @ Y_{max}$, Y_{max}, and Y_i) (see Tables 2, 3, 4 and 5). $X @ Y_{max}$ is the optimum amendment concentration that resulted in the maximum response (Y_{max}). For lognormal curves, values were determined from the 1st derivative of the response curve, such that $f'(X)=0$. Y_i is the calculated response at the highest amendment concentration tested. Significant differences in the calculated parameters ($X @ Y_{max}$, Y_{max}, and Y_i) between trial/treatment combinations were tested by ANOVA with Holm-Sidak *post-hoc* testing

using PROC MIXED (SAS, Vers 9.3). Inhibition values reported throughout the text were calculated as the percent difference between Y_i and Y_{max}.

Results

Leaf Tissue Colony diameter exhibited a log-normal relationship in response to the leaf tissue amendment of the culture medium (Fig. 1a). The amount of leaf tissue required to reach maximum colony growth ($X @ Y_{max}$) was significantly lower for the oak tissue than for the bay laurel (Table 2). However, the maximum colony size did not differ significantly between the three plant-amended media (Table 2). Increasing the amount of leaf tissue inhibited colony growth, with the largest percent difference between Y_i and Y_{max} observed with the two oak species (Table 2).

Similar to colony diameter, zoospore production showed a log-normal response to leaf tissue amendment of the culture medium (Fig. 2a). However, maximum zoospore production differed between the bay laurel and oak amendments, with nearly five-fold more zoospore production with bay laurel-containing medium (Table 3). Inhibition was noted at the highest concentration tested for all three species, with about 40 % inhibition in zoospore production for the bay laurel treatment and nearly 100 % for the oaks (Table 3).

The amount of ELISA-detected elicitin in the samples amended with leaf tissue (Fig. 3a) was similar to the pattern observed for zoospore production. For example, maximum elicitin secretion was nearly 2.5-fold higher with bay laurel than with the oaks; and inhibition was only about 10 % with bay laurel, as compared to approximately 50 % with the oaks (Table 4).

Plant and Commercial Sterol Amendments The foliage sterol extracts included a variety of sterol and non-sterol compounds: phytol, heptacosane, nonacosane, hentricontane, α-sitosterol, and β-sitosterol (Table 1). Although a statistical comparison

Table 2 *Phytophthora ramorum* colony diameter in response to various amendments. For each of three trials, response curves were fitted to each species/compound using PROC NLIN (SAS Vers 9.3). Values are the mean and SD of three independent trials. Values with different letters are significantly different ($P<0.05$). X @ Y_{max} units are the same as those defined in the source column, Y_{max} and Y_i units are mm

Source	Species/Compound	Form of fitted curve	X @ Y_{max}[a]	Y_{max}[b]	Y_i[c]
Foliage (mg ml^{-1})	bay laurel	Log-normal	0.54 (0.09) a	43.6 (1.1) a	38.5 (2.9) a
	black oak	Log-normal	0.28 (0.03) b	44.3 (0.4) a	15.4 (2.6) b
	white oak	Log-normal	0.24 (0.03) b	41.7 (1.7) a	17.3 (2.2) b
Foliar sterols (µg ml^{-1})	bay laurel	Log-normal	1.53 (0.29) a	41.3 (1.2) a	36.0 (1.1) a
	black oak	Log-normal	1.64 (0.18) a	42.3 (1.9) a	35.5 (2.7) a
	white oak	Log-normal	1.44 (0.29) a	42.4 (2.4) a	35.2 (1.5) a
Sterols (µM)	β-sitosterol	Log-normal	7.52 (1.91) b	39.2 (1.0) a	33.5 (2.8) b
	cholesterol	Log-normal	21.71 (2.51) a	40.3 (1.9) a	39.3 (1.9) a
	ergosterol	Exponential rise	nd	nd	42.5 (1.0) a
	stigmastanol	Exponential rise	nd	nd	40.5 (1.4) a
	stigmasterol	Exponential rise	nd	nd	40.9 (0.8) a
Foliar tannins (µg ml^{-1})	bay laurel	Exponential decay	0	41.2 (2.2) a	23.2 (1.8) a
	black oak	Exponential decay	0	40.4 (1.7) a	21.8 (1.7) a
	white oak	Exponential decay	0	39.9 (1.5) a	22.5 (2.1) a

[a] X @ Y_{max} is the optimum amendment concentration yielding the maximum response. For lognormal curves, values were determined from the 1st derivative of the response curve, such that $f'(X)=0$. *nd* not determined.

[b] Y_{max} is the maximum response value

[c] Y_i is the calculated response at the highest amendment concentration tested (foliage: $X=5$ mg dry extract ml^{-1}; foliar sterols: $X=10$ µg dry extract mL^{-1}; foliar tannins: $X=500$ µg dry extract mL^{-1}; sterols: $X=50$ µM)

among the three species is not possible due to a lack of replicate trees, the three extracts exhibited a range of sterol (α- and β-sitosterol) contents (1,745, 612, and 712 µg g^{-1} DW) for the bay laurel, black oak, and white oak extracts, respectively

Table 3 *Phytophthora ramorum* zoospore production in response to various amendments. For each of three trials, response curves were fitted to each species/compound using PROC NLIN (SAS Vers 9.3). Values are the mean and SD of three independent trials. Values with different letters are significantly different ($P<0.05$). X @ Y_{max} units are the same as those defined in the source column, Y_{max} and Y_i units are spores ml^{-1}

Source	Species/Compound	Form of fitted curve	X @ Y_{max}[a]	Y_{max}[b]	Y_i[c]
Foliage (mg g^{-1})	bay laurel	Log-normal	1.54 (0.18) a	695 (83) a	411 (52) a
	black oak	Log-normal	0.41 (0.08) b	134 (48) b	0 (0) b
	white oak	Log-normal	0.35 (0.08) b	126 (49) b	1 (1) b
Foliar sterols (µg ml^{-1})	bay laurel	Log-normal	3.51 (0.15) a	298 (13) a	212 (18) a
	black oak	Log-normal	3.65 (0.70) a	292 (27) a	248 (39) a
	white oak	Log-normal	3.55 (0.28) a	293 (22) a	205 (22) a
Sterols (µM)	β-sitosterol	Log-normal	13.94 (3.17) b	1,254 (206) a	197 (82) c
	cholesterol	Log-normal	35.88 (5.37) a	283 (282) b	176 (134) c
	ergosterol	Linear	≥ 50	≥ 866	866 (108) b
	stigmastanol	Linear	≥ 50	≥ 2,754	2,754 (333) a
	stigmasterol	Linear	≥ 50	≥ 2,594	2,594 (199) a
Foliar tannins (µg ml^{-1})	bay laurel	Exponential decay	0	138 (13) a	30 (9) a
	black oak	Exponential decay	0	134 (8) a	25 (6) a
	white oak	Exponential decay	0	130 (7) a	27 (8) a

[a] X @ Y_{max} is the optimum amendment concentration yielding the maximum response. For lognormal curves, values were determined from the 1st derivative of the response curve, such that $f'(X)=0$

[b] Y_{max} is the maximum response value

[c] Y_i is the calculated response at the highest amendment concentration tested (foliage: $X=5$ mg dry extract ml^{-1}; foliar sterols: $X=10$ µg dry extract ml^{-1}; foliar tannins: $X=500$ µg dry extract mL^{-1}; sterols: $X=50$ µM)

Table 4 ELISA-detectable *Phytophthora ramorum* elicitin production in response to various amendments. For each of three trials, response curves were fitted to each species/compound using PROC NLIN (SAS Vers 9.3). Values are the mean and SD of three independent trials.

Values with different letters are significantly different ($P<0.05$). $X @ Y_{max}$ units are the same as those defined in the source column, Y_{max} and Y_i units are nM.

Source	Species/Compound	Form of fitted curve	$X @ Y_{max}$[a]	Y_{max}[b]	Y_i[c]
Foliage(mg ml^{-1})	bay laurel	Log-normal	1.92 (1.82) a	26.2 (1.7) a	23.2 (1.8) a
	black oak	Log-normal	0.31 (0.55) b	10.8 (2.1) b	5.3 (1.2) b
	white oak	Log-normal	0.25 (0.46) b	11.1 (1.9) b	6.2 (1.6) b
Foliar sterols (µg ml^{-1})	bay laurel	Log-normal	15.34 (3.83) a	18.6 (1.0) a	18.5 (2.0) a
	black oak	Log-normal	17.62 (6.81) a	20.6 (1.3) a	19.2 (1.7) a
	white oak	Log-normal	15.89 (4.30) a	19.2 (1.6) a	18.8 (2.4) a
Sterols (µM)	β-sitosterol	Log-normal	10.21 (2.77)	34.4 (1.8)	15.7 (2.8) a
	cholesterol	Exponential rise	nd	nd	25.3 (2.5) b
	ergosterol	Exponential rise	nd	nd	26.7 (2.5) b
	stigmastanol	Exponential rise	nd	nd	43.2 (2.6) c
	stigmasterol	Exponential rise	nd	nd	42.7 (3.1) c
Foliar tannins (µg ml^{-1})	bay laurel	Exponential decay	0	15.5 (2.2) a	4.2 (1.8) a
	black oak	Exponential decay	0	17.6 (1.9) a	4.0 (0.8) a
	white oak	Exponential decay	0	16.1 (2.4) a	3.1 (1.6) a

[a] $X @ Y_{max}$ is the optimum amendment concentration yielding the maximum response. For lognormal curves, values were determined from the 1st derivative of the response curve, such that $f'(X)=0$

[b] Y_{max} is the maximum response value

[c] Y_i is the calculated response at the highest amendment concentration tested (foliage: $X=5$ mg dry extract ml^{-1}; foliar sterols: $X=10$ µg dry extract mL^{-1}; foliar tannins: $X=500$ µg dry extract mL^{-1}; sterols: $X=50$ µM)

(Table 1). Similar to the leaf tissue amendments, there was a log-normal relationship between leaf sterol amendments and *P. ramorum* colony diameters (Fig. 1b), zoospore production (Fig. 2b), or elicitin secretion (Fig. 3b). For all three parameters, similar patterns were observed regardless of the origin of the plant sterol extract (Tables 1, 2 and 3). Similar to the leaf tissue amendments, inhibition was observed at the highest concentrations tested. For example, colony diameters were inhibited

15.3 % (Table 2), zoospore production 24.7 % (Table 3), and elicitin secretion 3.1 % (Table 4). The effect of plant sterols on elicitin gene expression, as measured by RT-qPCR, showed a log-normal response curve for all three plant species (Fig. 4a). The curves did not differ among species for any of the descriptors ($X @ Y_{max}$, Y_{max}, or Y_i, Table 5). Similar to colony diameter and zoospore production, elicitin gene expression was inhibited at the highest concentration of foliar sterols (Table 5).

Table 5 *Phytophthora ramorum* ram-*a*2 elicitin gene expression in response to various amendments. For each of three trials, response curves were fitted to each species/compound using PROC NLIN (SAS Vers 9.3). Values are the mean and SD of three independent trials.

Values with different letters are significantly different ($P<0.05$). $X @ Y_{max}$ units are the same as those defined in the source column, Y_{max} and Y_i units are dimensionless

Source	Species/Compound	Form of fitted curve	$X @ Y_{max}$[a]	Y_{max}[b]	Y_i[c]
Foliar sterols (µg ml^{-1})	bay laurel	Log-normal	4.03 (1.82) a	0.82 (0.12) a	0.61 (0.11) a
	black oak	Log-normal	3.54 (1.44) a	0.77 (0.17) a	0.57 (0.08) a
	white oak	Log-normal	3.98 (2.06) a	0.80 (0.09) a	0.64 (0.05) a
Foliar tannins (µg ml^{-1})	bay laurel	Linear	nd	nd	0.75 (0.09) a
	black oak	Linear	nd	nd	0.64 (0.15) a
	white oak	Linear	nd	nd	0.79 (0.10) a

[a] $X @ Y_{max}$ is the optimum amendment concentration yielding the maximum response. For lognormal curves, values were determined from the 1st derivative of the response curve, such that $f'(X)=0$

[b] Y_{max} is the maximum response value

[c] Y_i is the calculated response at the highest amendment concentration tested (foliage: $X=5$ mg dry extract ml^{-1}; foliar sterols: $X=10$ µg dry extract mL^{-1}; foliar tannins: $X=500$ µg dry extract mL^{-1}; sterols: $X=50$ µM)

Fig. 1 *Phytophthora ramorum* colony diameter in response to **a** ground foliage, **b** foliar sterol extracts, **c** foliar tannin extracts, **d** β-sitosterol and cholesterol, and **e** ergosterol, stigmastanol and stigmasterol. Each point is the average (*N*=9) value for all three trials
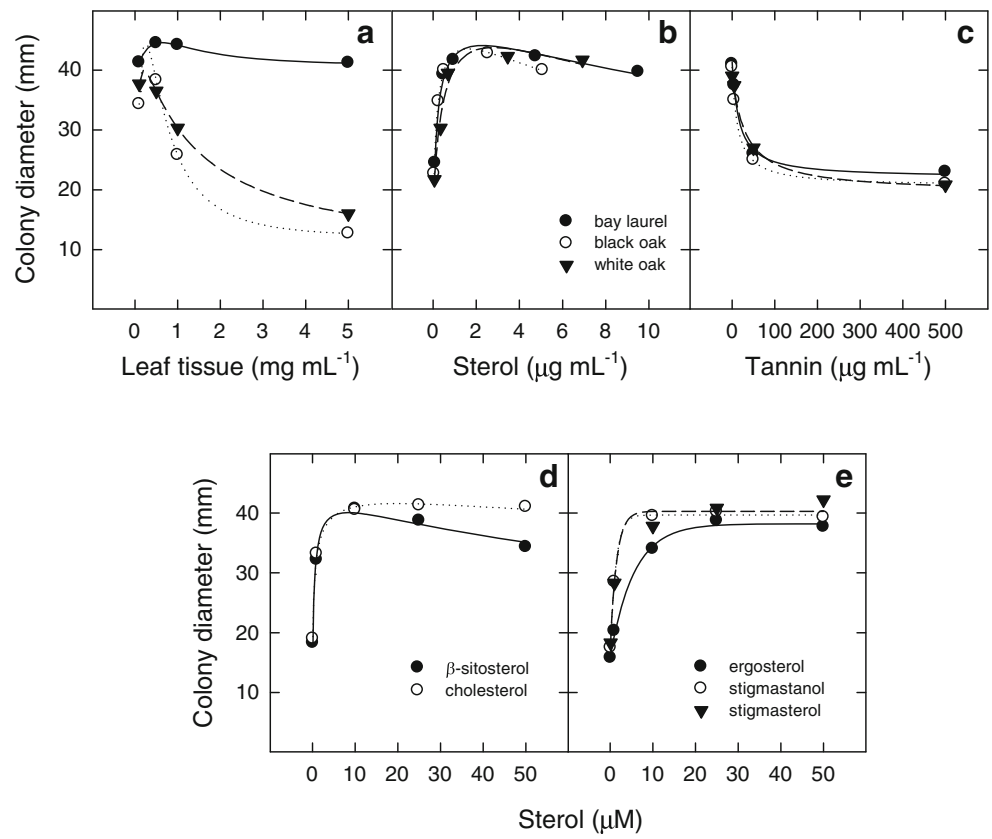
Fig. 2 *Phytophthora ramorum* zoospore production in response to **a** ground foliage, **b** foliar sterol extracts, **c** foliar tannin extracts, **d** β-sitosterol and cholesterol, and **e** ergosterol, stigmastanol and stigmasterol. Each point is the average (*N*=9) value for all three trials
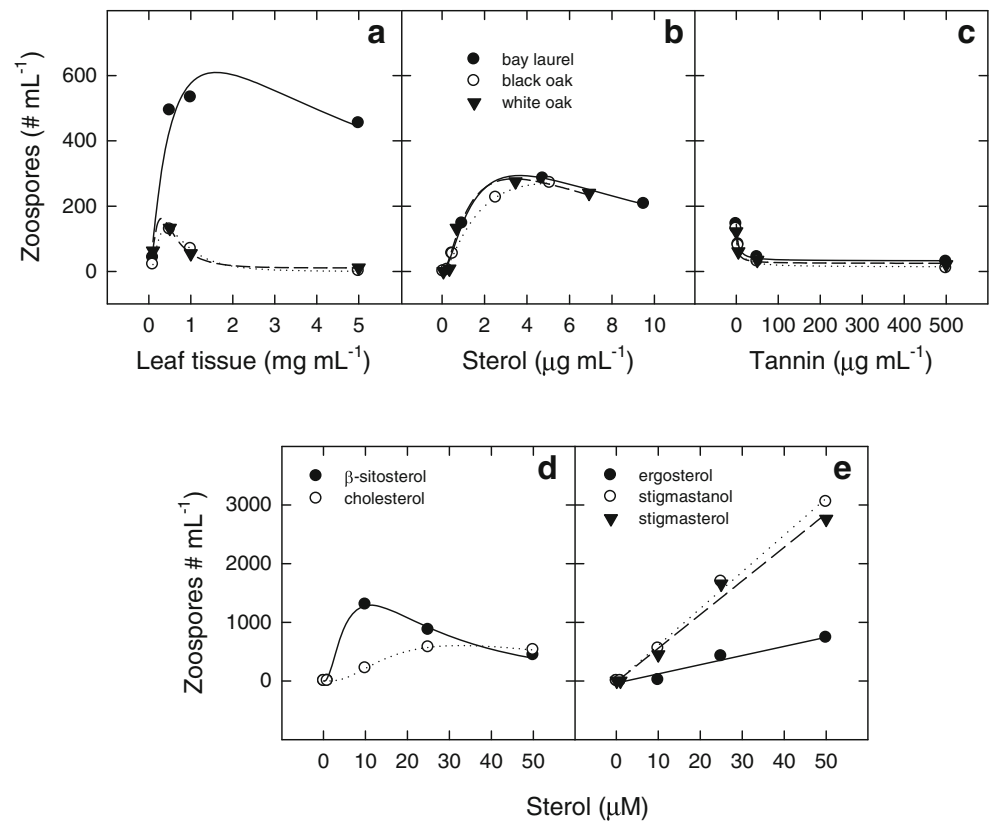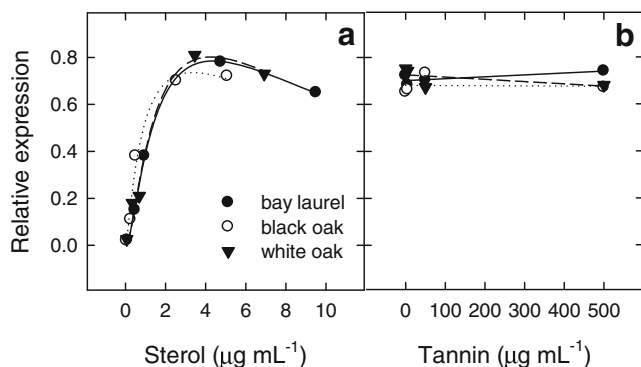
Fig. 3 ELISA-detectable *Phytophthora ramorum* elicitin secretion in response to **a** ground foliage, **b** foliar sterol extracts, **c** foliar tannin extracts, **d** β-sitosterol and cholesterol, and **e** ergosterol, stigmastanol and stigmasterol. Each point is the average (*N*=9) value for all three trials
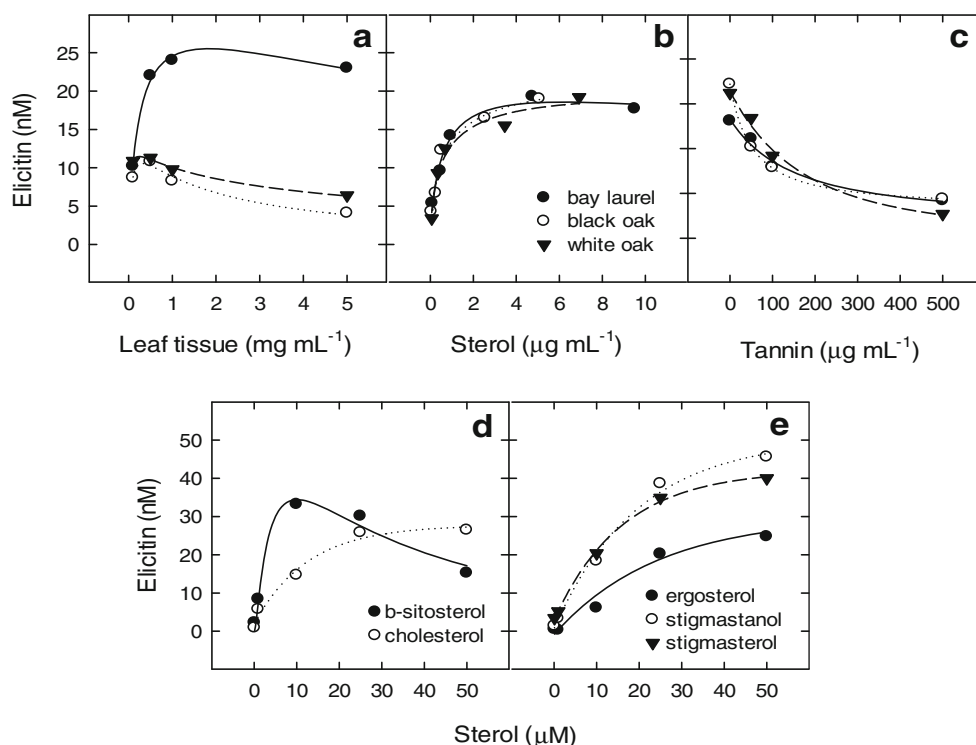
Qualitative differences in the effect of sterols on *P. ramorum* growth and sporulation were tested using a variety of commercially available sterols (β-sitosterol, cholesterol, egrosterol, stigmastanol, and stigmasterol). For colony diameters, the response to sterol amendments was either log-normal (β-sitosterol, cholesterol) or exponential rise (ergosterol, stigmastanol, and stigmasterol) (Fig. 1d,e, Table 1). The only sterol with any inhibitory effect on colony size was β-sitosterol at high concentrations (Table 2). The different sterols had different effects on production of zoospores (Fig. 2d,e, Table 3). For example, a decline in zoospore production with high levels of sterols was observed for β-sitosterol (84.2 % inhibition) and cholesterol (37.8 % inhibition) (Table 3); whereas, zoospore production

exhibited a positive linear response to ergosterol, stigmastanol, and stigmasterol. The effect of sterols on elicitin secretion was similar to the trends observed for zoospore production, except that β-sitosterol inhibited elicitin production by 54.3 %, while no inhibition was noted for any of the four other sterols (Fig. 3d,e, Table 4).

Tannins Gravimetric estimates of leaf tannin content after Sephadex LH-20 purification indicated that the concentration of tannin was highest for black oak, moderate for white oak, and lowest for bay laurel (Table 1). Total phenolics (Folin-Denis), gallotannin (rhodanine), and condensed tannin (acid butanol) assays all showed similar patterns, with the highest concentrations present in black oak, followed by white oak, and the lowest in bay laurel (Table 1). Tannin amendments had an inhibitory effect on all parameters measured, except elicitin gene expression (Figs. 1c, 2c, 3c, 4b). With increasing tannin concentrations, a significant decline was seen for colony diameters (Table 2), zoospore production (Table 3), and ELISA-detected elicitin secretion (Table 4) across all three species. In contrast, *P. ramorum* ram-α2 gene expression remained constant at all levels of tannin amendments tested, suggesting that elicitin gene expression is not affected by tannin concentrations (Fig. 4, Table 5).

Colony diameter and zoospore production were highly correlated with the amount of ELISA-detected elicitin across all treatment and trials (Fig. 5). It is worth noting that these highly significant relationships hold true across the three plant species tested despite differing sterol and

Fig. 4 RT-qPCR Expression analysis of *ramorum* ram-α2 elicitin gene expression in response to **a** foliar sterol extracts and **b** foliar tannin extracts. Each point is the average (*N*=9) value for all three trials

tannin concentrations and compositions. Furthermore, the amount of elicitin required to maximize colony diameter growth is much lower than is required for zoospore production (Fig. 5). Zoospore production did not appear to reach its maximal level in this study.

Discussion

Phytophthora ramorum growth and sporulation exhibited two general trends relative to sterols: (i) the response curves differed depending upon the type of sterol present and (ii) high levels of sterols could lead to a decline in *P. ramorum* growth and sporulation, which is consistent with other *Phytophthora* spp. (Elliott et al. 1966; Haskins et al. 1964; Hendrix 1970; Leal et al. 1964; Nes and Stafford 1983). The mechanistic basis for the varying response curves, or sterol 'discrimination' is unknown; however, it may be dependent upon differences in sterol uptake and metabolism (Mikes et al. 1998; Nes and Stafford 1983, 1984) or gene expression (Yousef et al. 2009). Regardless of the mechanism, it is clear that optimal *P. ramorum* growth and sporulation was achieved at different levels for each of the sterols tested in this study. For example, *P. ramorum* zoospore production was maximized at ca. 14 µM for β-sitosterol and ca. 36 µM for cholesterol; whereas, a maximum was not reached for ergosterol, stigmastanol, or stgmasterol at the concentrations tested. The decline in *P. ramorum* growth and the amount of ELISA-detectable elicitins at high concentrations of either β-sitosterol or cholesterol is consistent with the ability of sterols to stimulate a down-regulation in elicitin gene expression in *P. sojae* (Yousef et al. 2009).

In this study, gene expression was not evaluated for each sterol individually; however, the foliar sterol extracts, comprised of mainly α- and β-sitosterol, did diminish elicitin gene expression at concentrations above ca. 4 µg ml^{-1}. It is possible that the sterol-dependent down-regulation of *Phytophthora* spp. elicitin genes balances the need to evade plant detection, with the acquisition of sterols required for sporulation. For example, cholesterol is a major component of leaf surface lipids (Noda et al. 1988); whereas, β-sitosterol is often the major sterol component within plant tissues (Gunstone et al. 1994). Elicitin gene expression, and sterol acquisition, may be maximized on the leaf surface where sporulation occurs, and minimized within plant cells to avoid host defense responses. The strong relationship between sterol acquisition and *Phytophthora* growth and sporulation, and the apparent discrimination between sterols, suggests that plant sterol profiles may be useful predictors of plant susceptibility or sporulation of *Phytophthora* spp. However, in a study by Hazel et al. (1988), the sterol profiles of potato leaves were not suitable predictors of field resistance to *P. infestans* or its sporulation potential, indicating that other factors are involved.

Tannins may be one such factor, as evidenced by our data showing that tannin-enriched foliage extracts (Sephadex LH20 purified) negatively influence *P. ramorum* growth and colonization. The mechanistic basis for this inhibition, however, is unknown. One plausible mechanism may be the binding of elicitins by tannins, thus inhibiting sterol uptake and therefore inhibiting *Phytophthora* growth and colonization. Tannins, by definition, have the potential to bind and precipitate proteins; and the tannin-enriched extracts used in this study have the ability to bind and precipitate *P. ramorum* elicitins in solution (Manter, *unpublished data*). In the studies reported here, the tannin-enriched extracts reduced the amount of ELISA-detectable water-soluble elicitins (i.e., flooding of PSM agar plates) but did not influence elicitin gene expression. We hypothesize that tannin-elicitin binding may remove the elicitins from solution and/or interfere with the elicitin-antibody binding required for the ELISA-based detection. In
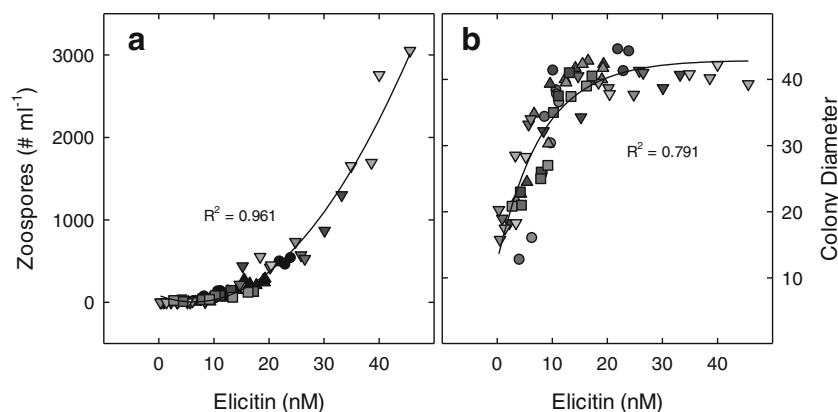
Fig. 5 Relationship between ELISA-detectable *Phytophthora ramorum* elicitin secretion and **a** zoospore production or **b** colony diameter growth. Each point is the average (*N*=9) value for all three trials. Symbol shapes denote amendment type—leaf tissue (*circles*), foliar sterol extracts (*upward triangles*), commercial sterols (*downward triangles*), foliar tannin extracts (*squares*).; symbol colors (gray intensity, from dark to light) denote plant species—bay (*black*), black oak (*gray*), white oak (*no color*) or commercial sterols—β-sitosterol (*black*), cholesterol (*dark gray*), ergosterol (*gray*), stigmastanol (*light gray*), stigmasterol (*no color*)

either case, the amount of elicitin available for sterol binding (i.e., 'functional' elicitin) may be reduced leading to a decrease of *P. ramorum* growth and sporulation. We are unaware of any other studies that have examined the potential role of tannins on *P. ramorum* growth and sporulation; however, some simple phenolics (e.g., tyrosol, gallic acid) have direct antimicrobial activity against *P. ramorum* (Ockels et al. 2007). The mechanistic basis associated with their antimicrobial activity has not been studied; however, it is interesting to note that these simple phenolics are the precursors for the polyphenolic tannins (Bianco and Savolaninen 1997) and may be indicative of increased tannin production in infected tissue.

The studies reported here were conducted *in vitro*; therefore, it is unknown to what degree elicitins, tannins, and sterols interact *in planta*. However, there is some evidence that this interaction may occur. For example, both tannins (Evert 2006) and elicitins (Brummer et al. 2002; Osman et al. 2001) associate with plant cell walls suggesting that their interaction is spatially possible; although, whether this truly happens can only be found through further *in planta* research. Furthermore, additional work with other plant tissues, i.e., bole/stems, needs to be pursued, as *P. ramorum* colonization is often limited to the bole in a variety of oak species. Due to the limitations of the tannin assays used in this study, additional studies will also be required to determine the specific compound(s) in the tannin-enriched extract or any other plant compound(s) that interfere with elicitin activity and subsequent *P. ramorum* growth and sporulation. In summary, our data indicate that the amount of 'functional' elicitin (i.e., ELISA-detectable and presumably the amount of elicitin available to bind sterols), not necessarily elicitin gene expression, is influenced by both sterol and tannin contents and is highly correlated with *P. ramorum* growth and sporulation *in vitro*.

References

Behrman EJ, Gopalan V (2005) Cholesterol and plants. J Chem Educ 82:1791–1793

Bianco MA, Savolaninen H (1997) Phenolic acids as indicators of wood tannins. Sci Total Environ 203:79–82

Brummer M, Arend M, Fromm J, Schlenzig A, Osswald WF (2002) Ultrastructual changes and immunocytochemical localization of the elctin quercinin in *Quercus robur* L. roots infected with *Phytophthora quercina*. Physiol Mol Plant Pathol 61:109–120

Elliott CG, Hendrie MR, Knights BA (1966) The sterol requirement of *Phytophthora cactorum*. J Gen Microbiol 42:425–35

Evert RF (2006) Esau's Plant Anatomy. Wiley, New Jersey

Fleischmann F, Koehl J, Portz R, Beltrame AB, Osswald W (2005) Physiological changes of *Fagus sylvatica* seedlings infected with *Phytophthora citricola* and the contribution of its elicitin "citricolin" to pathogenesis. Plant Biol 7:650–8

Folin O, Denis W (1912) On phosphotungstic-phosphomolbdic compounds as color reagents. J Biol Chem 12:239–245

Goheen EM, Hansen E, Kanaskie A, Osterbauer N, Parke J, Pscheidt J, Chastagner G (2006) Sudden oak death and *Phytophthora ramorum*. Oregon State University Extension Service

Gonzalez RA, Parks LW (1981) Lack of specificity in accumulation of sterols by *Phytophthora cactorum*. Lipids 16:384–388

Grant BR, Greenway W, Whatley FR (1988) Metabolic changes during development of Phytophthora palmivora examined by Gas Chromatography/Mass Spectrometry. J Gen Microbiol 134:1901–1911

Grunwald NJ, Goss EM, Press CM (2008) *Phytophthora ramorum*: a pathogen with a remarkably wide host range causing sudden oak death on oaks and ramorum blight on woody ornamentals. Mol Plant Pathol 9:729–40

Gunstone FD, Harwood JL, Padley FB (1994) The Lipid handbook. New York: Chapman and Hall. XIII, 722, 551 p

Hagerman AE, Butler LG (1981) The specificity of proanthocyanidin-protein interaction. J Biol Chem 256:4494–4497

Haskins RH, Tulloch AP, Mirceitich RG (1964) Steroids and the stimulation of sexual reproduction of species of *Pythium*. Can J Microbiol 10:187–195

Hazel WJ, Bean GA, Goth RW (1988) Relationship of potato leaf sterols to development of potato late blight caused by *Phytophthora infestans* on U.S. potato clones and breeding lines. Plant Dis 72:203–205

Hendrix JW (1970) Sterols in growth and reproduction of fungi. Annu Rev Phytopathol 8:111–130

Hoitink HAJ, Schmitthenner AF (1974) Relative prevalence and virulence of *Phytophthora* species involved in Rhododendron root rot. Phytopathology 64:1371–1374

Inoue KH, Hagerman AE (1988) Determination of gallotannin with rhodanine. Anal Biochem 169:363–9

Ivanova DG, Singh BR (2003) Nondestructive FTIR monitoring of leaf senescence and elictin-induced changes in plant leaves. Biopolymers 72:79–85

Jeong W-S, Lachance PA (2001) Phytosterols and fatty acids in Fig (*Ficus carica* var. Mission) fruit and tree components. *J.* Food Sci 66:278–281

Kamoun S, Klucher KM, Coffey MD, Tyler BM (1993) A gene encoding a host-specific elicitor protein of *Phytophthora parasitica*. Mol Plant-Microbe Interact 6:573–581

Latte KP, Kolodziej H (2000) Antifungal effects of hydrolysable tannins and related compounds on dermatophytes, mould fungi and yeasts. Z Naturforsch C 55:467–72

Leal JA, Friend J, Holliday P (1964) A factor controlling sexual reproduction in *Phytophthora*. Nature 203:545–546

Manter DK, Kelsey RG, Karchesy JJ (2007a) Antifungal activity of extracts and select compounds in the heartwood of seven western conifers toward *Phytophthora ramorum*. J Chem Ecol 33:2133–2147

Manter DK, Kelsey RG, Karchesy JJ (2007b) Photosynthetic declines in *Phytophthora ramorum*-infected plants develop prior to water stress and in response to exogenous application of elicitins. Phytopathology 97:850–6

Manter DK, Kolodny EH, Hansen EM, Parke JL (2010) Virulence, sporulation, and elicitin production in three clonal lineages of *Phytophthora ramorum*. Physiol Mol Plant Pathol 74:317–322

Mikes V, Milat M-L, Ponchet M, Panabieres F, Ricci P, Blein J-P (1998) Elicitins excreted by Phytophthora are a new class of sterol carrier proteins. Biochem Biophys Res Comm 245:133–139

Nagle AM, McPherson BA, Wood DL, Garbelotto M, Bonello P (2011) Relationship between resistance to *Phytophthora ramorum*

and constitutive phenolic chemistry in coast live oak. For Pathol 41:464–469

Nelson KE, Pell AN, Doane BI, Giner-Chavez BI, Schofield P (1997) Chemical and biological assays to evaluate bacterial inhibition by tannins. J Chem Ecol 23:1175–1194

Nes WD, Stafford AE (1983) Evidence for metabolic and functional discrimination of sterols by *Phytophthora cactorum*. Proc Natl Acad Sci USA 80:3227–3231

Nes WD, Stafford AE (1984) Side-chain structural requirements for sterol-induced regulation of *Phytophthora cactorum* physiology. Lipids 19:544–9

Nes WD, Patterson GW, Bean GA (1980) Effect of steric and nuclear changes in steroids and triterpenoids on sexual reproduction in *Phytophthora cactorum*. Plant Physiol 66:1008–11

Noda M, Tanaka M, Seto Y, Aiba T, Oku C (1988) Occurrence of cholesterol as a major sterol compenent in leaf surface lipids. Lipids 23:439–444

Ockels FS, Eyles A, McPherson BA, Wood DL, Bonello P (2007) Phenolic chemistry of coast live oak response to *Phytophthora ramorum* infection. J Chem Ecol 33:1721–32

Osman H, Vauthrin S, Mikes V, Milat M-L, Panabieres F, Marais A, Brunie S, Maume B, Ponchet M, Blein J-P (2001) Mediation of elicitin activity on tobacco is assumed by elicitin-sterol complexes. Mol Biol Cell 12:2825–2834

Panabieres F, Marais A, le Berre J, Penot I, Fournier D, Ricci P (1995) Characterization of a gene cluster of *Phytophthora cryptogea* which codes for elicitins, proteins inducing a hypersensitive-like response in tobacco. Mol Plant-Microbe Interact 8:996–1003

Pernollet J-C, Sallantin M, Sallé-Tourne M, Huet J-C (1993) Elicitin isoforms from seven Phytophthora species: comparison of their physio-chemical properties and toxicity to tabacco and other plant species. Physiol Mol Plant Pathol 42:53–67

Porter LJ, Hrstich LN, Chan BG (1986) The conversion of procyanidins and prodelphinidins to cyanidin and delphinidin. Phytochemistry 25:223

Rizzo DM, Garbelotto M, Hansen EM (2005) *Phytophthora ramorum*: integrative research and management of an emerging pathogen in California and Oregon forests. Annu Rev Phytopathol 43:309–35

Shahidi F, Naczk M (1995) Food phenolics. Technomic Publishing Co., Lancaster

Sivakumaran S, Molan AL, Meagher LP, Kolb B, Foo LY, Lane GA, Attwood GA, Fraser K, Tavendale M (2004) Variation in antimicrobial action of proanthocyanidins from Dorycnium rectum against rumen bacteria. Phytochemistry 65:2485–97

Strumeyer DH, Malin MJ (1975) Condensed tannins in grain sorghum: isolation, fractionation, and characterization. J Agric Food Chem 23:909–14

Vauthrin S, Mikes V, Milat M-L, Ponchet M, Maume B, Osman H, Blein J-P (1999) Elicitins trap and transfer sterols from micelles, liposomes and plant plasma membranes. Biochim Biophys Acta Protein Struct Mol Enzymol 1419:335–342

Vleeshouwers VGAA, van Dooijeweert W, Govers F, Kamoun S, Colon LT (2000) The hypersensitive response is associated with host and nonhost resistance to *Phytophthora infestans*. Planta 210:853–864

Yousef LF, Yousef AF, Mymryk JS, Dick WA, Dick RP (2009) Stigmasterol and cholesterol regulate the expression of elicitin genes in *Phytophthora sojae*. J Chem Ecol 35:824–32